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Characterisation of pectin subunits released by an optimised combination of enzymes

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Abstract

Pectins from sugar beet, lime and apple were degraded by a rhamnogalacturonan hydrolase associated or not with pectin methylesterases and side chain degrading enzymes (galactanase and arabinanase). The composition of the enzymatic mixture was optimised by following the reaction by viscosimetric means. The reaction products were fractionated by ion exchange chromatography. Treatment with all the enzymes released four fractions: (1) 227–247 mg/g of initial pectins and corresponded to neutral sugars from the side chains; (2,3) represented together 184–220 mg/g of pectins and corresponded to rhamnogalacturonan; (4) 533–588 mg/g of pectins and corresponded to homogalacturonan. Lime pectins have the shortest rhamnogalacturonan regions. The molar masses of homogalacturonans were in the range of 16,000–43,400 g/mol according to the origin of pectins, corresponding to degrees of polymerisation of 85–250. The mode of action of the enzymes used is also discussed.

Keywords: Pectins; Enzymatic degradation; Rhamnogalacturonan hydrolase; Pectin methylesterases; Homogalacturonan

1. Introduction

Pectins are one of the main polysaccharides in the primary cell walls of dicots, and contain mainly galacturonic acid (GalA) and neutral sugars in varying proportions. They exhibit physico-chemical properties such as gelling capacity, explaining their large use in food industry.[1] Pectins are characterised by a backbone of α-(1 → 4)-linked GalA residues which are partly methyl- or acetyl-esterified. They also contain some associated neutral sugars, typically L-rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), and more rarely D-xylose (Xyl), and D-glucose (Glc). They are generally described as an alternance of 'smooth' regions, consisting of homogalacturonans, and 'hairy' regions, consisting of a rhamnogalacturonan backbone carrying side chains, typically α-(1 → 5)-linked arabinans or β-(1 → 4)-linked galactans.[1] They often carry some substituents such as methyl-ester on the carboxylic group of GalA or acetyl group on the O-2 and/or O-3 of GalA. The degree of methylation (DM) and degree of acetylation (DAc) are defined as the percentage of GalA esterified with methanol or acetic acid, respectively.

Many of the functionalities of pectins are related to their chemical structure. The relevant parameters include the amounts of GalA and neutral sugars, the amounts and distribution of substituents (methoxyl and acetyl groups), and the molar mass.[2] The ratio between 'smooth' and 'hairy' regions may also influence the pectin properties, as well as the number and/or size of side chains, of which the role is still an open question.[3] Thibault et al.[4] obtained the 'smooth' regions of beet, citrus and apple (de-esterified) pectins by acidic hydrolysis. However, these conditions were likely to degrade the galacturonan backbone, leading to underestimation of the molar mass values. Moreover, since pectins have to be de-esterified prior to the acidic treatment, the role of DM and DAc cannot be studied.

Abbreviations: DAc, degree of acetylation; DM, degree of methylation; dp, degree of polymerisation; Endo-A, endo-arabinanase; Endo-G, endo-galactanase; HPSEC-MALLS, high-performance size exclusion chromatography–multi angle laser light scattering; PME, pectin methylesterase; Rg-ase, rhamnogalacturonan-hydrolase.

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These disadvantages could be overcome by the use of pure enzymes which have been proven to be powerful tools to determine the fine structure of pectins.[5–12] Due to the variety of monomers, substituents, and linkages, numerous enzymes are required to degrade pectins. Polygalacturonases (EC 3.2.1.15) are hydrolases that split off α-(1→4) linkages between two GalA residues. They are inverting enzymes and belong to the family 28 of glycoside hydrolases, as defined by Henrissat and Bairoch.[13] Pectin methylesterases (PME, EC 3.1.1.11, carbohydrate esterase family 8) release the methyl groups, α-(1→5) arabinanases (EC 3.2.1.99, family GH43) and β-(1→4) galactanases (EC 3.2.1.89, family GH53) are specific for α-(1→5) linkages between Ara residues, and β-(1→4) linkages between Gal residues, respectively. The rhamnogalacturonan hydrolase (Rg-ase A, family GH28, EC not defined) cleaved galactopyranosyluronic–rhamnopyranosyl linkages in the rhamnogalacturonan backbone.[14]

In this paper, we obtained (fragments of) side chains, (fragments of) rhamnogalacturonans and homogalacturonans by using rhamnogalacturonase (Rg-ase), arabinanase (Endo-A), galactanase (Endo-G), and pectin methylesterases from orange (O-PME) as well as from *Aspergillus aculeatus* (F-PME). The reaction products were quantified and analysed. This study will also give some information about the mode of action of the enzymes used

2. Results

Enzymatic hydrolysis of sugar beet pectins and analysis of the reaction products.—Sugar beet pectins were extracted with water from extrusion-cooked sugar beet pulp as already described.[15] These pectins were rich in GalA (513 mg/g, Table 1), highly acetylated (DAc = 33) and contained large amounts of neutral sugars, particularly Ara, in agreement with previously published data.[15,16] Different enzymes (Rg-ase, O-PME, F-PME, Endo-A, Endo-G) were tested for the hydrolysis of beet pectins and the reaction was followed by measuring the viscosity and plotting the reciprocal of specific viscosity as a function of time (Fig. 1). Rg-ase alone or in combination with O-PME induced a low decrease of specific viscosity. In contrast, when F-PME was added to Rg-ase, the decrease in viscosity was much larger. When the Rg-ase was used in combination with Endo-

Table 1
Yields and chemical composition of extrusion-cooked sugar beet pulp, water-extractable pectins and fractions obtained by anion exchange chromatography (AEC), after enzymatic degradation by Rg-hydrolase, PMEs, Endo-A and Endo-G

	Extruded pulp	Pectins	After enzymatic degradation and AEC			
			1	2	3	4
Yield		143[a]	247[b]	154[b]	66[b]	533[b]
[NaCl] (M)			0	0	0.11	0.28
Composition (mg/g)						
Rha	14	19	1	104	198	12
Ara	97	136	844	262	259	17
Xyl	8	0	13	0	traces	2
Gal	32	48	42	0	92	25
Glc	172	8	44	0	0	0
GalA	176	513	0	633	434	838
Acetyl groups (DAc[c])	33 (56)	58 (33)	nd[e]	nd	nd	105 (38)
Methyl groups (DM[d])	14 (42)	43 (46)	nd	nd	nd	1 (7)
Proteins	101	1	nd	nd	nd	nd
Ashes	40	49	nd	nd	nd	nd
Molar masses[f]						
⟨Mw⟩ (⟨dpw⟩)						16,040 (84)
⟨Mn⟩ (⟨dpn⟩)						13,560 (71)

[a] mg/g of extruded pulp.

[b] mg/g of recovered pectins.

[c] Degree of acetylation.

[d] Degree of methylation.

[e] nd, not determined

[f] ⟨Mw⟩, weight-average molar mass; ⟨dpw⟩, weight-average degree of polymerisation; ⟨Mn⟩, number-average molar mass; ⟨dpn⟩, number-average degree of polymerisation.

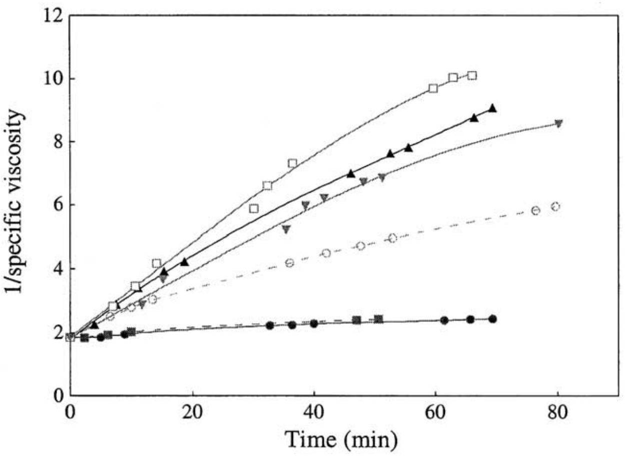

Fig. 1. Time changes in specific viscosity of reaction mixture containing sugar beet pectins and various enzymes alone or in admixture: (●) Rg-ase; (■) Rg-ase + O-PME; (○) Rg-ase + F-PME; (▼) Rg-ase + Endo-A + Endo-G; (▲) Rg-ase + O-PME + Endo-A + Endo-G; (□) Rg-ase + F-PME + Endo-A + Endo-G.

A and Endo-G, the hydrolysis was more efficient than with Rg-ase + F-PME. The degradation of pectins was even increased by addition of O-PME or F-PME to this mixture and the viscosity plateaued after 60 min of incubation. No insoluble fraction was released from sugar beet pectins, whatever the enzymes used. The reciprocal of the specific viscosity was linearly related to the reaction time in the initial steps of degradation (Fig. 1), suggesting that the enzymes randomly degraded rhamnogalacturonan.

The initial pectin as well as the reaction products obtained after incubation with each enzyme or enzymatic combination were fractionated by anion exchange (DEAE Sepharose CL6B) and size exclusion chromatographies (Sepharose CL6B). Anion exchange chromatography gave a small unbound fraction (25 mg/g of recovered material) and a bound fraction eluted in one thin peak with 0.19 M NaCl, showing that the pectin was quite homogeneous in terms of charge density (Fig. 2A). Size exclusion chromatography (Fig. 3A) revealed three main populations, one eluting at the void volume and containing more neutral sugars than uronic acids, one eluting at $K_{av} \approx 0.4$ and containing more uronic acids than neutral sugars, and one eluting at $K_{av} \approx 0.6$ and mainly composed of uronic acids. Hydrolysis by Rg-ase alone did not significantly modify the anion exchange profile (Fig. 2B). Size exclusion chromatography (Fig. 3B) revealed that the amount of material eluting at the void volume decreased whereas the K_{av} of the major peak was still ≈ 0.6. The decrease in viscosity (Fig. 1) could therefore be ascribed to the degradation of the highest molar mass molecules of pectin. When O-PME was added to Rg-ase, the anion exchange chromatography (Fig. 2C) showed a peak eluting with a higher concentration of NaCl (0.28 M) than in the control showing to the demethylation of the polymer. The size exclusion profile was similar to that obtained from Rg-ase alone (Fig. 3C), showing that the demethylation occurred without depolymerisation. When F-PME was used in place of O-PME, a very thin peak eluted with 0.1 M NaCl containing both uronic acids and neutral sugars (Fig. 2D), ascribed to rhamnogalacturonan oligomers produced by Rg-ase after

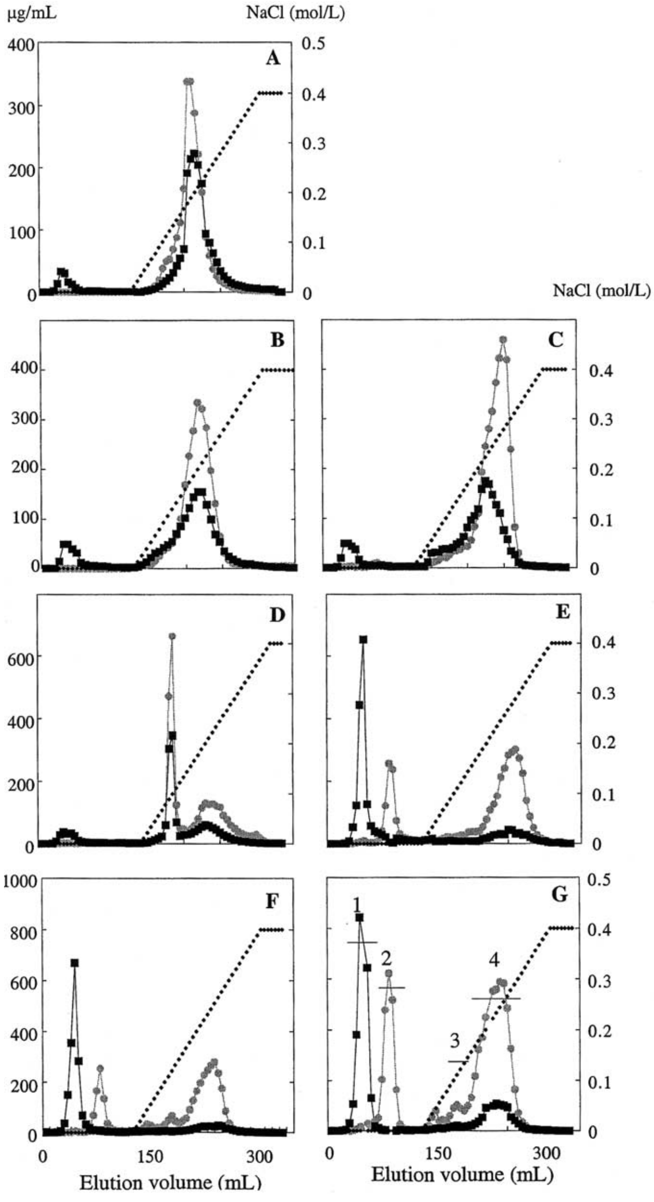

Fig. 2. Anion exchange chromatographies (DEAE Sepharose CL6B) eluted by a NaCl gradient in sodium succinate buffer (pH 4.5) of hydrolysis media containing sugar beet pectins incubated with various enzymes alone or in admixture: **A**, control; **B**, Rg-ase; **C**, Rg-ase + O-PME; **D**, Rg-ase + F-PME; **E**, Rg-ase + O-PME + Endo-A + Endo-G; **F**, Rg-ase + F-PME + Endo-A + Endo-G; **G**, Rg-ase + O-PME + F-PME + Endo-A + Endo-G. (■) neutral sugars (µg/mL); (●) uronic acids (µg/mL); (◆) [NaCl] (mol/L).

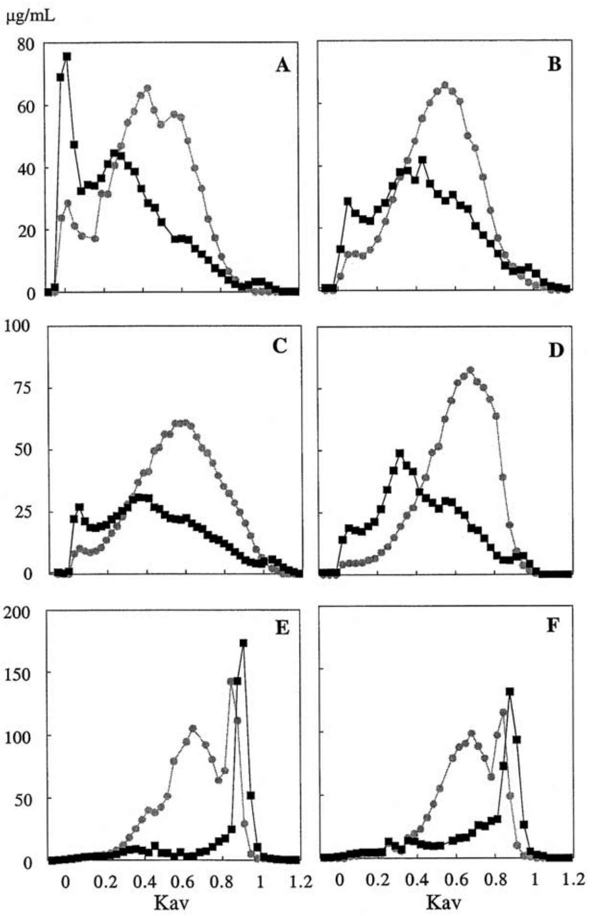

Fig. 3. Size exclusion chromatographies (Sepharose CL6B) eluted by sodium succinate buffer (pH 4.5) of hydrolysis media containing sugar beet pectins incubated with various enzymes alone or in admixture: **A**, control; **B**, Rg-ase; **C**, Rg-ase + O-PME; **D**, Rg-ase + F-PME; **E**, Rg-ase + O-PME + Endo-A + Endo-G; **F**, Rg-ase + F-PME + Endo-A + Endo-G. (■) neutral sugars (µg/mL); (●) uronic acids (µg/mL).

demethylation by F-PME. These results suggested that O-PME was not able to de-esterify the rhamnogalacturonan backbone while F-PME was. On the size exclusion chromatography profile (Fig. 3D), the main acidic peak eluted at higher K_{av} (0.7), due to the depolymerisation already indicated by viscosimetry (Fig. 1).

By adding Endo-A and Endo-G to Rg-ase and PMEs, most of the neutral sugars eluted as an unbound fraction (Figs. 2E and 2F). A peak of uronic acids appeared before the gradient. The molarity of NaCl necessary for eluting the most acidic fraction was higher after action of O-PME (0.29 M) than after action of F-PME (0.25 M), showing that the blockwise demethylation allowed the polymer to be more strongly bound to the column. Fractionation on size exclusion chromatography of the reaction mixture containing Rg-ase, Endo-A and Endo-G in admixture with either O-PME (Fig. 3E) or F-PME (Fig. 3F) showed two peaks, one eluting around K_{av} 0.7 and corresponding to 55% of recovered sugars and the second one eluting near the total volume. The first fraction was mainly composed of GalA (molar ratio neutral sugars/GalA = 0.14), whereas the second fraction contained neutral oligosaccharides. Anion exchange fractionation of the reaction products released by all the enzymes in admixture (Fig. 2G) showed (i) a non-retained fraction (fraction 1), composed of neutral sugars and representing 247 mg/g of pectins, (ii) a fraction composed of uronic acids (fraction 2, 154 mg/g of pectins), (iii) a minor retained peak eluted with 0.11 M NaCl (fraction 3, 66 mg/g of pectins), and (iv) a last peak mainly composed of uronic acids eluting with about 0.28 M NaCl (fraction 4, 533 mg/g of pectins). Their composition is shown on Table 1. Fraction 1 contained exclusively neutral sugars, mainly Ara originating from side chains. Fraction 2 was mainly composed of uronic acids with some Rha and Ara, and corresponded to short rhamnogalacturonic oligomers still carrying Ara units. Fraction 3 contained GalA and Rha (molar ratio GalA/Rha = 2.4), Ara and Gal. Fraction 4 was very rich in GalA (838 mg/g), and was almost devoid of neutral sugars (<7 mg/g). It was highly acetylated (DAc = 38). The low value of DM (DM = 7) confirmed the action of the PMEs. Therefore, these four fractions could be considered as representative of the pectin subunits, the fraction 1 corresponding to (fragments of) side chains, fraction 2 + fraction 3 to (fragments of) rhamnogalacturonan, and fraction 4 to homogalacturonan. Molar mass of the fraction 4 was determined by HPSEC-MALLS (Table 1). Number- and weight-average degrees of polymerisation (dp) of 71–84 with a low polydispersity (1.19) were calculated taken into account the DAc. Similar dp was previously found after acidic treatment of beet pectin.[4]

Enzymatic hydrolysis of apple and lime pectins.— Both pectins were from commercial origin. Initial values of DM and $\langle Mw \rangle$ of apple pectins were 69 and 146,000 g/mol, respectively. Anion exchange profile (Fig. 4A) yielded 117 mg neutral fraction/g pectin and 883 mg acidic fraction/g pectin eluted with 0.22 M NaCl. The neutral fraction contained 505 mg Gal/g, 402 mg Glc/g and 46 mg Ara/g (Table 2). The high amounts of Gal and Glc may be explained by the presence of starch and/or the addition of lactose for the standardisation of this commercial pectin.[1] The acidic fraction was largely composed of GalA (857 mg/g), but also contained some neutral sugars (67 mg Gal/g, 27 mg Rha/g, 21 mg Ara/g, and 15 mg Xyl/g). The lime pectins (DM = 72, $\langle Mw \rangle$ = 133,000 g/mol) were highly pure since only 51 mg/g of the initial material were not retained on the column, corresponding mainly to arabinans and galactans.

The enzyme combination selected to degrade sugar beet pectins was used for apple and lime pectins as well

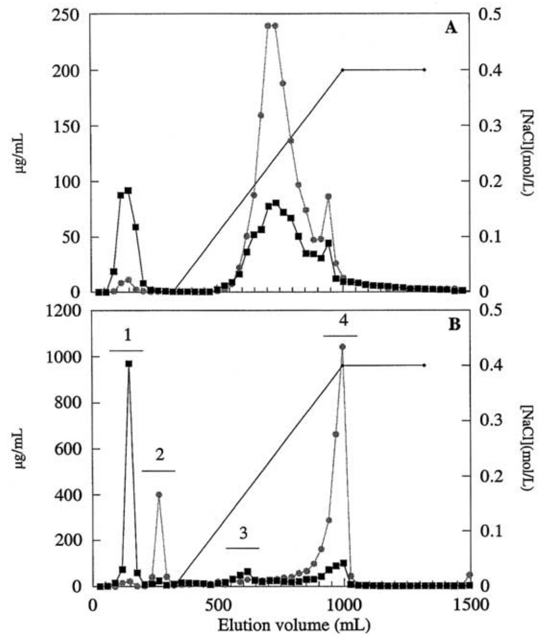

Fig. 4. Anion exchange chromatographies (DEAE Sepharose CL6B) eluted by a NaCl gradient in sodium succinate buffer (pH 4.5) of apple pectins; **A**, initial pectins; **B**, after degradation with Rg-hydrolase + O-PME + F-PME + Endo-A + Endo-G. (■) neutral sugars (μg/mL); (●) uronic acids (μg/mL); (◆) [NaCl] (mol/L).

as the fractionation means of the products. The two elution profiles were very similar to that obtained from sugar beet pectins (Figs. 4B and 5B) as well as the yields of each fraction (Tables 2 and 3). From apple pectins (Table 2), the fractionation yielded 227 mg fraction 1/g of initial material. Fractions 2 and 3 represented 107 mg/g and 77 mg/g of pectins, respectively while the fraction 4 was the major one (588 mg/g of initial material). The non-retained fraction contained about 55% of initial neutral sugars, mainly Glc and Gal. Assuming that the bulk of Glc originates from starch, it was possible to subtract the content in Glc (617 mg/g of fraction 1) and to calculate new yields in each fraction: Fraction 1, 101 mg/g, Fraction 2, 125

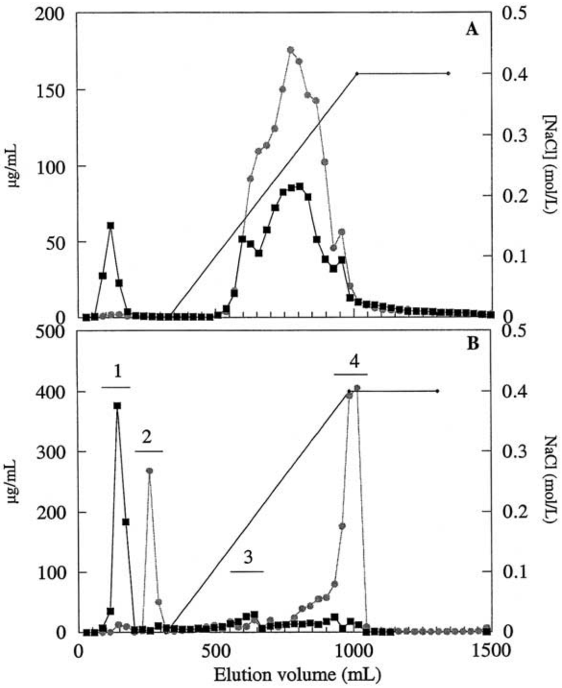

Fig. 5. Anion exchange chromatographies (DEAE Sepharose CL6B) eluted by a NaCl gradient in sodium succinate buffer (pH 4.5) of lime pectins; **A**, initial pectins; **B**, after degradation with Rg-hydrolase + O-PME + F-PME + Endo-A + Endo-G. (■) neutral sugars (μg/mL); (●) uronic acids (μg/mL); (◆) [NaCl] (mol/L).

Table 2
Yield, composition and molar masses of apple pectins and degradation products after enzymatic degradation and anion exchange chromatography (AEC)

	Pectins	After AEC		After enzymatic degradation and AEC			
		1	2	1	2	3	4
Yield (mg/g)		117	883	227	107	77	588
[NaCl] (M)		0	0.23	0	0	0.17	0.4
Composition (mg/g)							
Rha	46	2	27	4	91	231	18
Ara	32	46	21	57	52	54	11
Xyl	27	20	15	40	3	17	35
Gal	160	505	68	203	198	148	24
Glc	120	402	9	617	2	10	10
GalA	611	11	857	14	619	484	882
Molar masses							
⟨Mw⟩ (⟨dpw⟩)	146,000						43,400 (247)

⟨Mw⟩, weight-average molar mass; ⟨dpw⟩, weight-average degree of polymerisation.

mg/g, Fraction 3, 90 mg/g, Fraction 4, 684 mg/g. However, the content in Gal could not be deducted similarly since it may arise either from side chains or from lactose. Fractions 2 and 3 differed by their neutral sugar content, especially in Rha. The molar ratio GalA/Rha decreased from 5.8 in the fraction 2 to 1.8 in the fraction 3. Fraction 4 was mostly composed of GalA (882 mg/g). The peak was very narrow and eluted with a high ionic strength (NaCl = 0.4 M) due to the demethylation. Fraction 4 also contained 35 mg Xyl/g, in agreement with the presence of xylogalacturonan in apple pectins.[4,18] A weight-average molar mass of 43,400 g/mol was measured for apple homogalacturonans (Table 2). Thus, the dp_w (247) was much higher than that found for beet homogalacturonan.

The enzymatic degradation of lime pectins followed by anion exchange chromatography yielded 4 fractions very similar to those obtained from the other pectins (Table 3). The fraction 1 represented 236 mg/g of pectins. By considering the amount of unbound fraction before enzymatic hydrolysis, this fraction 1 corresponded to 185 mg neutral sugars/g of pectin. It contained about 80% of initial neutral sugars, mainly Ara and Gal. The fraction 2 (131 mg/g) corresponded to short oligomers of rhamnogalacturonans (molar ratio GalA/Rha = 5.7) still carrying neutral sugars (mainly Gal). In the fraction 3 (83 mg/g), the molar ratio GalA/Rha was 0.8 and the amount of neutral sugars was very low. The fraction 4 was the major one (549 mg/g) and exhibited mainly GalA with a low quantity of Rha. This fraction could be a homogalacturonan. A weight-average molar mass of 34,000 g/mol was found for lime homogalacturonan, corresponding to a dp_w of 193.

3. Discussion

Pectins from beet, apple, and lime were degraded by a combination of enzymes and the reaction products were fractionated by anion exchange chromatography and analysed.

Our results gave some information about the mode of action of the enzymes. O-PME and F-PME are known to act blockwise and randomly, respectively.[19,20] Our data suggested that they differed by their specificity. Indeed, it is likely that F-PME was able to remove methoxyl groups from the rhamnogalacturonan region whereas O-PME was not. The blockwise de-esterification by O-PME may be hindered either by Rha or by side chains, both preventing the enzyme to glide along the rhamnogalacturonan backbone. These results were in disagreement with those of Matsuura[21] who showed that a PME from mandarine peels acted on rhamnogalacturonan as well as homogalacturonan. As shown by Limberg et al.,[22] a plant PME needs a certain number of free carboxyl groups to form an enzyme–substrate complex. Indeed, Rha and GalA in rhamnogalacturonan are strictly in alternance,[23] and it is not possible to find adjacent GalA residues allowing the enzyme to act and to glide along the substrate.

The Rg-ase is known to be hindered by acetyl groups[14] explaining its low activity when used alone on beet pectins (Fig. 1). However, its efficiency in the presence of F-PME suggested that it could also be inhibited by methyl groups. This result was not in agreement with that obtained by Schols et al.[14] who observed a degradation of modified apple 'hairy' regions by Rg-ase before as well as after chemical saponification. However, no information was given on the efficiency of hydrolysis in both cases.

Table 3
Yield, composition and molar masses of lime pectins and degradation products after enzymatic degradation and anion exchange chromatography (AEC)

	Pectins	After AEC		After enzymatic degradation and AEC			
		1	2	1	2	3	4
Yield (mg/g)		51	948	236	131	83	549
[NaCl] (M)		0	0.26	0	0	0.19	0.4
Composition (mg/g)							
Rha	38	4	12	7	83	381	10
Ara	51	466	25	296	65	30	5
Xyl	2	9	2	15	13	9	3
Gal	75	355	30	271	112	36	8
Glc	7	78	3	24	31	1	1
GalA	823	24	823	40	565	385	926
Molar masses							
⟨Mw⟩ (⟨dpw⟩)	152,000						34,000 (193)

⟨Mw⟩, weight-average molar mass; ⟨dpw⟩, weight-average degree of polymerisation.

Our results gave also some information on the structure of the pectins. The three pectins led to similar elution patterns after enzymatic hydrolysis and anion exchange chromatography, whatever the source. The fractions recovered can be considered as the subunits of pectins. It can be noticed that the distribution of each main sugar in the four fractions was similar for beet, lime and apple pectins: Ara and Gal were largely present in the fractions 1 whereas they were almost absent in the fractions 4; more than 70% of initial rhamnose was present in the fractions $2+3$; the fractions 4 contained around 80% of initial GalA.

The comparison of the values presented in Tables 1–3 shows that all the pectins contained about 240 mg of neutral sugar/g (fraction 1), 180–220 mg of rhamnogalacturonan/g (fractions $2+3$), and 530–590 mg of homogalacturonan/g (fraction 4). However in the case of apple pectin, the results had to be corrected by the contamination of co-extracted starch. In the four fractions, GalA was shared as follows: the fraction 1 contained up to 1.5% of initial GalA, rhamnogalacturonan ($=$ fractions $2+3$) contained 16.5–22% of initial GalA, whereas homogalacturonan ($=$ fractions 4) contained $\approx 85\%$ of initial GalA, except in lime pectin where it reached only 61.8%. The proportion of GalA in rhamnogalacturonan was much lower in onion rhamnogalacturonans where it represented only 2.6%, as calculated after degradation of the pectins with an exopolygalacturonase and an exopolygalacturonate lyase.[24] In contrast, previous studies of apple pectins established that 92–95% of GalA residues were in the homogalacturonans.[17,25] In these studies, the determination of GalA from homogalacturonan was made after chemical β-elimination and endopolygalacturonase degradation. In our study, endopolygalacturonase was not used and the fraction resistant to the enzymatic hydrolysis was quantified as homogalacturonan.

The Rha content, calculated as a percentage of the initial content, reached 0.7–3.3% in the fractions 1, 21.8–44.8% in the fractions 2, 36.6–63.4% in the fractions 3, and 11–27.4% in the fractions 4. If we consider the fractions $2+3$, the Rha proportion was in the range of 71–85%. As the fractions $2+3$ corresponded to rhamnogalacturonan, the proportion of Rha present in rhamnogalacturonan was in the same range for all the pectins. Lime pectins were the less sensitive to the hydrolysis (21.8% of initial rhamnose in the fraction 2, 63.4% of initial amount in the fraction 3). It could be related to the length of the rhamnogalacturonic backbone. In this case, lime pectins would have the shortest rhamnogalacturonan regions.

The molar ratio GalA/Rha in the fractions 2 and 3 was around 5 and 1, respectively and was similar for the three pectins. So, the fractions 3 corresponded to a true rhamnogalacturonan with a strict alternance of Rha and GalA.[23] A ratio of 1 was also found in a fraction released from beet pectins by a Rg-ase.[12] The degradation of apple modified 'hairy' regions by Rg-ase as well as treatment of carrot cell wall polysaccharides with endopectin lyase also resulted in fractions with a molar ratio GalA/Rha around 1.[7,14] Our three pectins have different initial content in Rha but exhibited similar amounts of rhamnogalacturonan (fractions $2+3 \approx$ 200 mg/g) with similar ratio GalA/Rha. The remaining Ara and Gal in the rhamnogalacturonan fraction could be ascribed to the presence of feruloyl groups on these sugars,[26] that could hinder the enzyme accessibility. According to Zhan et al.,[27] 85% of Rha was present in a strictly repeating [GalA-Rha] alternance in commercial citrus pectins. The remaining 15% could be randomly distributed in the homogalacturonan or could form rhamnogalacturonan–homogalacturonan junction. They do not form short rhamnogalacturonic sequences that could be recognised by Rg-ase, as 4 [-GalA-($1 \rightarrow 2$) Rha-] patterns in the chain are necessary for hydrolysis.[28] However, endopolygalacturonase digestion of commercial citrus pectins did not release final product originating from the homogalacturonan and containing isolated Rha.[27]

The homogalacturonan released from beet, lime and apple pectins had a weight-average dp around 84, 193 and 247, respectively (fractions 4). Thus, the chain length of 72–100 GalA residues of acid resistant homogalacturonans[4] was underestimated. In this study, the homogalacturonan was obtained as a precipitate due to the conditions used (de-esterified pectins in acidic conditions). The acid-insoluble material obtained by acidic hydrolysis contained almost exclusively GalA (more than 98% mol). On the opposite after enzymatic degradation, the fractions 4 from lime, beet and apple pectins still contained 28, 56 and 98 mg of neutral sugars/g, respectively and were soluble in the conditions used (pH 4.5).

Degradation of pectins by PMEs, Rg-ase, Endo-A and Endo-G in admixture, followed by anion exchange chromatography allowed quantification of their different subunits as long as co-extracted polysaccharide, such as starch, is excluded. Whole 'smooth' regions were released by the action of Rg-ase alone on apple cell wall suggesting that homogalacturonan and rhamnogalacturonan were part of the same complex pectic molecule.[29] Similarly, our results were compatible with a pectin model consisting of 'smooth' and 'hairy' regions covalently bound, and with 'hairy' regions carrying neutral sugar side chains.

4. Experimental

Pectins.—Apple and lime pectins were from Danisco Cultor (Braband, DK). Sugar beet pulp (Générale Sucrière, France) was treated in a twin-screw extruder Clextral BC 45, as described elsewhere.[15]

Table 4
Sugar composition of the substrates used for the determination of enzymatic activities

	Galactan	Arabinan	Rhamnogalacturonan	Homogalacturonan
Rha	0.2	0.9	19.3	0.9
Ara	2.2	88.6	1.2	0.1
Man	0.4	0	0.2	0
Xyl	0	0	0.2	0
Gal	95.6	6.8	24.3	0.2
Glc	0	0	0	0
GalA	1.4	3.7	54.8	98.4

Content in each sugar is given in % mol.

Enzymes.—Pectin methylesterase from orange peel (O-PME) was purchased from Sigma Chemicals (L'Isle d'Abeau, France). Recombinant fungal pectin methylesterase (F-PME, Swiss-Prot entry Q12535) and rhamnogalacturonan hydrolase (Rg-ase A, Swiss-Prot entry Q00018) were provided by Novozymes (Copenhagen, DK). They were originally cloned from *Aspergillus aculeatus* and expressed in *A. oryzae*. The enzymes were dissolved in 0.05 M succinate buffer pH 4.5 at 1 mg/mL for PMEs and 10 mg/mL for Rg-ase, and were dialysed against the same buffer before use.

Enzymatic assays.—Enzymatic activities are expressed in nkatals, 1 nkat being the amount of enzyme which releases 1 nmol of reducing ends per s under the defined conditions.

The enzymes were assayed towards substrates from commercial origin (galacturonan, Sigma Chemicals) or from laboratory collection: homogalacturonan,[4] arabinan,[30] rhamnogalacturonan,[31] and galactan.[32] Compositions are shown in Table 4. The activities were calculated from the increase in reducing ends,[33] assayed in microplates[34] using appropriate sugars for standard curves. Reaction mixtures contained 0.9 mL substrate (0.1 g/L in 0.05 M succinate buffer, pH 4.5) and 0.1 mL enzyme solutions at 40 °C. PME activity was measured towards lime pectin (DM 72) by titrimetry.[35] Acetylesterase activity was measured by spectrophotometric determination of *p*-nitrophenol[36] released from *p*-nitrophenyl acetate (Sigma Chemicals).

Arabinanase and galactanase purification.—Endo-A and Endo-G were purified to homogeneity from Pectinex AR (Novozymes, Copenhagen, DK), originating from *A. niger*. All the purification steps were carried out at 4 °C. After extensive dialysis against water, the preparation was precipitated with 70% saturation of ammonium sulphate. The precipitate was dissolved in water, dialysed and subjected to preparative isoelectric focusing (Rotofor Bio-Rad) in the presence of carrier ampholytes BioLyte pH 2.5–5. The fractions were assayed towards galactan, arabinan, and galacturonan. Arabinanase and galactanase active fractions were then submitted to chromatography on a Superdex 75 column (60 × 1.6 cm) using a Fast Protein Liquid Chromatography system (Amersham Biosciences, Orsay, France). The column was eluted with 0.05 M acetate buffer pH 4.5 at a flow rate of 0.3 mL/min. The arabinanase fraction was dialysed against water, freeze-dried and solubilised in 0.05 M acetate buffer pH 5. The galactanase fraction still contained polygalacturonase activity. After conditioning the sample in 1 M ammonium sulphate, the galactanase was purified using chromatography on Sepharose 6B cross-linked with divinyl sulfone (DVS).[32] The DVS Sepharose column (16 × 1.6 cm) was eluted by 5 column volumes of 0.05 M acetate buffer pH 4 and then 3.5 column volumes of 1 M acetate buffer pH 4. Activities measured after each

Table 5
Purification of Endo-A and Endo-G from Pectinex AR

	Volume (mL)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification factor
Endo-A					
Crude preparation	42	9470	124.7	75.9	
Ammonium sulphate precipitation	28	8618	100.1	86.1	1.1
Preparative electrofocusing	11	5512	44.7	123.3	1.6
Superdex 75	3.6	1920	2.9	662.1	8.7
Endo-G					
Crude preparation	42	44169	124.7	354.2	
Ammonium sulphate precipitation	28	34870	100.1	348.4	1.0
Preparative electrofocusing	11	19348	44.7	432.8	1.2
Superdex 75	62	7992	8.5	940.2	2.7
DVS Sepharose	31.5	1598	0.5	3196.0	9.0

purification step are summarised in Table 5. The galactanase was free of arabinanase and vice versa. Both enzymes were devoid of polygalacturonase as well as glycosidase or esterase activity. Some properties of these two enzymes such as molar mass, pI, and final products, were previously reported.[30,32]

Enzymatic hydrolysis of pectins.—200 mg pectins were dissolved in 24 mL 0.05 M succinate buffer pH 4.5 and were degraded by 5.6 mL of a enzymatic mixture containing 25 nkat F-PME, 25 nkat O-PME, 3.6 nkat Rg-ase, 122 nkat galactanase, 107 nkat arabinanase. No activity towards homogalacturonan was found in the enzyme mixture. The reaction mixture was incubated for 1.5 h at 40 °C and boiled before to be loaded on chromatography column.

Viscosity analysis.—The viscosity of the reaction mixture was measured using an automated viscosimeter (AVS 310, Schott Geräte, Germany) fitted with an Ostwald capillary tube (2 mL, diameter 0.4 mm). Specific viscosities were calculated as $\eta_{\text{spe}} = (t - t_o)/t_o$, where t = flow time of reaction mixture, and t_o = flow time of buffer, 77.35 s.

Chromatographies.—Anion exchange chromatography was performed at room temperature on DEAE-Sepharose CL6B column (14 × 2.6 cm) equilibrated with 0.05 M succinate buffer pH 4.5. The reaction mixture was loaded and eluted at a flow rate of 0.7 mL/min with 2 column volumes succinate buffer, followed by 4 column volumes linear gradient up to 0.4 M NaCl in 0.05 M succinate buffer pH 4.5 and 2 column volumes of the same buffer containing 0.4 M NaCl.

Size exclusion chromatography was performed at room temperature on a Sepharose CL6B column (80 × 2.2 cm) equilibrated with 0.05 M succinate buffer pH 4.5. The total volume of reaction mixture was loaded and eluted at 0.4 mL/min with the same buffer. Fractions (5 mL and 4 mL, for anion exchange and size exclusion chromatographies, respectively) were assayed for GalA and neutral sugars (see Analytical methods). Yields of all the chromatographies were in the range of 89–110%.

High-performance size exclusion chromatography (HPSEC) was performed at room temperature on two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ, exclusion limit 1.106 and 4.106 for pullulan, respectively) eluted at 0.6 mL/min with 0.05 M sodium nitrate containing 0.02% sodium azide. The column effluent was monitored using an on line multi angle laser light scattering (MALLS) detector (Mini Dawn, Wyatt Technology Corp., USA) and differential refractometer (Erma 7512, Japan). Molar masses were calculated with Astra 1.4 software using refractive index increment dn/dc = 0.146 g/mL.

Analytical methods.—Dry matter was determined by drying the sample at 120 °C for 2 h. All the data were expressed on a moisture-free basis. Ashes were weighed after overnight incineration of samples at 550 °C followed by 1 h at 900 °C. Individual neutral sugars were quantified after hydrolysis (6 h in 2 M H_2SO_4, 100 °C), derivatisation in their alditol acetates,[37] and analysis by gas liquid chromatography on a DB-225 fused-silica capillary column (J&W Scientific, Courtaboeuf, France; 30 m × 0.32 mm i.d.) mounted in a DI 200 chromatograph (Delsi Nermag Instruments, Argenteuil, France). For the sugar beet pulp, a prehydrolysis step (1 h in 72% H_2SO_4 at room temperature) was added. Uronic acid (as GalA) and neutral sugar (as Ara) contents were colorimetrically determined by the *m*-hydroxybiphenyl method[38] automated by Thibault[39] and orcinol method,[40] respectively, the later being corrected for interfering GalA. DM and DAc were calculated after hplc determination of methanol and acetic acid[41] released by alkaline de-esterification of pectins. Soluble proteins were measured colorimetrically,[42] with bovine serum albumin as standard. In sugar beet pulp, protein content (N × 6.25) was determined by micro-Kjeldahl procedure.

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